Amplified fragment length polymorphism and mitochondrial sequence data detect genetic differentiation and relationships in endangered southwestern U.S.A. ambersnails (*Oxyloma* spp.)

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Abstract: The Kanab ambersnail (*Oxyloma haydeni kanabensis*) is a federally endangered mollusc currently known to reside in two locations in the southwestern U.S.A. To determine the extent of within- and between-population genetic variation of this taxon, the amplified fragment length polymorphism (AFLP) technique was used to generate 110 genetic markers among individuals sampled from the two Kanab ambersnail populations and from the only two known southwestern populations of the Niobrara ambersnail (*Oxyloma haydeni haydeni*) in Utah and northern Arizona. Additional information was obtained from sequence data of cytochrome *b* and cytochrome oxidase I gene fragments. Results suggest high levels of differentiation among populations, as evidenced through the application of UPGMA (unweighted pair-group method with arthimetic averaging) clustering, *F* statistics, and Fisher's exact test. Various levels of within-population genetic diversity were observed among populations. Expected heterozygosities ranged from 0.239 to 0.086 under a model assuming Hardy–Weinberg genotypic proportions and ranged from 0.205 to 0.061 under an obligate-selfing completely homozygous model. Results from cluster analyses showed that one Kanab ambersnail population and one Niobrara ambersnail population were more similar than the two Kanab ambersnail populations studied (supported by >80% of bootstrap replicates). These findings were further supported through the phylogenetic analysis of both mitochondrial gene fragments. The data suggest that taxonomic designations need revision, an act that will likely affect the protected status of some of the populations.

Résumé : Le gastropode *Oxyloma haydeni kanabensis* est sur la liste fédérale des espèces menacées de mollusques et il habite en deux endroits dans le sud-ouest des États-Unis. Pour déterminer l'ampleur de la variation génétique au sein des populations et entre les populations de ce taxon, nous avons procédé à la technique du polymorphisme de la longueur des fragments amplifiés (AFLP) pour générer 110 marqueurs génétiques parmi les individus échantillonnés dans les populations d'*O. h. kanabensis* et des deux seules populations connues d'*Oxyloma haydeni haydeni* du Utah et du nord de l'Arizona. Des informations additionnelles ont été obtenues à partir de données sur les séquences des fragments des gènes cytochrome *b* et cytochrome oxydase I. Les résultats indiquent des différences importantes entre les populations, comme l'ont révélé les techniques de regroupement UPGMA, les valeurs de *F* et le test de Fisher. Divers niveaux de diversité génétique ont été observés au sein de chaque population. L'hétérozygotie théorique se situait entre 0,239 et 0,086 d'après un modèle basé sur les proportions génotypiques Hardy–Weinberg, et se situait entre 0,205 et 0,061 d'après un modèle à autofécondation obligée complètement homozygote. Les résultats des analyses de groupements indiquent qu'une population d'*O. h. kanabensis* et une population d'*O. h. haydeni* sont très semblables que les deux populations étudier d'*O. h. kanabensis* (d'après plus de 80 % de bootstraps répétés). Ces résultats sont de plus confirmés par une analyse phylogénétique des deux fragments de gènes mitochondriaux. Nos données semblent indiquer que les appellations taxonomiques nécessitent d'être révisées, un processus qui affectera sans doute le statut protégé de certaines des populations.

[Traduit par la Rédaction]

Introduction

The Kanab ambersnail (KAS; *Oxyloma haydeni kanabensis*) is a federally endangered succineid land snail that is presently known from two springs in the southwestern U.S.A. (Pilsbry and Ferriss 1911; Pilsbry 1948; Spamer and Bogan 1993). This taxon is restricted to Three Lakes (3L) near Kanab, Utah, and Vaseys Paradise (VP) in Grand Canyon

Received June 22, 1999. Accepted June 28, 2000.

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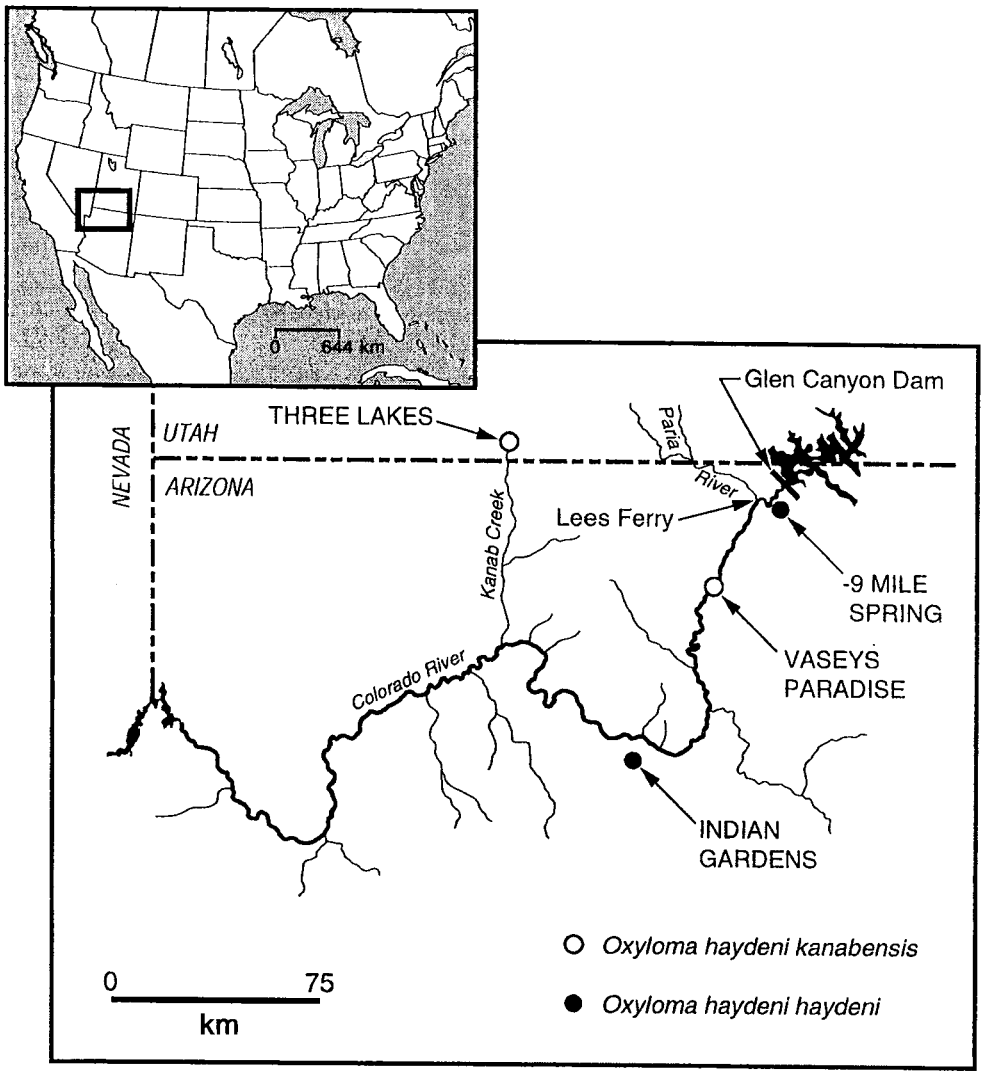
Fig. 1. Map of the locations of the four southwestern U.S.A. ambersnail populations studied.



National Park, Arizona (Fig. 1). KAS was proposed for emergency listing as an endangered species in 1991 (U.S. Fish and Wildlife Service 1991*a*, 1991*b*) and was subsequently listed in 1992 (U.S. Fish and Wildlife Service 1992*a*, 1992*b*). One additional KAS population formerly occurred in the Kanab, Utah, area but was apparently extirpated by desiccation of its habitat (Clarke 1991; U.S. Fish and Wildlife Service 1995). The 3L population in Utah occurs on a privately owned site. Neither the KAS nor any other *Oxyloma* species were observed at more than 125 other springs in the Grand Canyon region surveyed from 1991 to 1996 (Sorensen and Kubly 1997, 1998; Stevens et al. 1997*a*[2]), except for the Niobrara ambersnail (NAS; *Oxyloma haydeni haydeni*), which was present at Indian Gardens (IG) in Grand Canyon National Park and at –9 Mile Spring (–9M) upriver from Lees Ferry, Arizona (Fig. 1). While NAS occurs widely at latitudes from ~35° to 55°N (Harris and Hubricht 1982), the IG and –9M populations were not discovered until 1995 and 1996, respectively (L.E. Stevens and J.A. Sorensen, personal communication), and are the only NAS populations currently known in the southwestern U.S.A.

The objective of this study was to provide resource managers with information on levels of within- and among-population genetic variation of both endangered and nonendangered southwestern ambersnails. To accomplish this, the amplified fragment length polymorphism (AFLP) procedure (Vos et al. 1995) was used to generate molecular markers for analysis. Population-level relationships were also characterized using the AFLP data and DNA sequence information from regions of the cytochrome *b* (*Cytb*) and cytochrome oxidase subunit I (*CoxI*) genes.

Materials and methods

DNA isolation

DNA was isolated from snail foot tissue using a modification of the procedure described by Müllenbach et al. (1989). Specimens used for analysis were collected during the 1996 or 1997 growing

[2] L.E. Stevens, F.R. Protiva, D.M. Kubly, V.J. Meretsky, and J.R. Petterson, 1997*a*. The ecology of Kanab ambersnail (Succineidae: *Oxyloma haydeni kanabensis* Pilsbry, 1948) at Vaseys Paradise, Grand Canyon, Arizona. Report of the Glen Canyon Environmental Studies Program, U.S. Bureau of Reclamation, Flagstaff, Ariz.

season with permission from the U.S. Fish and Wildlife Service. Tissue was homogenized in a 1.5 mL microcentrifuge tube containing 460 μL extraction buffer (10 mM Tris, 50 mM EDTA, 1% SDS) followed by the addition of 30 μL proteinase K (10 mg/mL) and 10 μL DTT (0.1 M). Samples were incubated at 55°C for 2 h, after which 214.5 μL 5 M NaCl was added. Samples were further incubated for another 30 min at 55°C prior to pelleting cellular debris by centrifugation for 10 min at 12 000 rpm. After centrifugation, 600 μL of the supernatant was removed to a new microcentrifuge tube and mixed with an equal volume of chloroform. Samples were then incubated on an orbital shaker at 100 rpm for 30 min and centrifuged at 12 000 rpm for 10 min, after which 460 μL of the aqueous phase was removed and mixed with 460 μL of isopropanol. Samples were incubated at room temperature on an orbital shaker for 5 min to precipitate DNA, which was then collected via centrifugation at 12 000 rpm for 15 min. After a wash with 70% ethanol, pellets were resuspended in 50 μL TE (10 mM Tris, 0.1 mM EDTA, pH 8). DNA quantity and quality were evaluated visually on a 0.7% agarose gel through the use of ethidium bromide staining.

AFLP procedure

Genetic marker polymorphisms were resolved using the general procedure of Vos et al. (1995) with modifications described below. Analyses were conducted on 17 specimens from 3L, 23 specimens from IG, 21 specimens from VP, and 22 specimens from –9M. Restriction–ligation (RL) reactions were conducted using 50–500 ng template DNA in 1× RL buffer (10 mM Tris-acetate, 10 mM magnesium acetate, 10 mM potassium acetate, 5 mM DTT, pH 7.5) with 5 U of both *Eco*RI and *Mse*I. Samples were digested for 1 h at 37°C, followed by the addition of ligation reagents (1× RL buffer, 0.2 mM ATP, 1 U T_4 ligase, 5 pmol each of Keygene adapters 91M35 and 91M36 for *Eco*RI overhangs, 50 pmol each of Keygene adapters 92A18 and 92A19 for *Mse*I ends). Ligation reactions were incubated at 37°C for 3 h and then diluted 1:10 in TE in preparation for polymerase chain reaction (PCR).

The first selective PCR was conducted using the methods described in Travis et al. (1996) with adenine as the selective nucleotide. The second selective amplification was conducted using the following selective nucleotides: *Eco*ACG–*Mse*ACG, *Eco*ACC–*Mse*ACC, *Eco*ACC–*Mse*AGC, *Eco*AGC–*Mse*ACC, *Eco*AGC–*Mse*AGC. *Eco*RI + 3 primers were radiolabeled in a 25 μL kinase reaction containing 250 ng *Eco*RI + 3 primer, 5 U T4 polynucleotide kinase, 50 μCi [γ-^{32}P]ATP (1 Ci = 37 Gbq), 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl, and 1 mM 2-mercaptoethanol. Kinase reactions were incubated at 37°C for 30 min, followed by 10 min at 72°C. PCR followed in 10-μL reactions containing 2.5 ng labeled *Eco*RI + 3 primer, 15 ng *Mse*I + 3 primer, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 U Taq DNA polymerase, and 2.5 μL of a 1:20 dilution of template DNA from the first selective amplification. Cycling parameters for this reaction were the same as those in Travis et al. (1996). Gel analysis followed according to the methods of Vos et al. (1995) with the modifications given in Travis et al. (1996). Data were collected by recording the presence or absence of polymorphic markers for each individual; data were pooled over all markers and primer combinations used. Sizes of markers were estimated on the basis of their comparison with markers generated from a sequencing reaction of bacteriophage M13.

DNA sequencing

To further resolve the phylogenetic relationships of the four populations studied, sequence data were obtained from the *CoxI* and *Cytb* genes of two individuals from each of the four southwestern ambersnail populations. PCR primers were designed for amplification and subsequent sequencing reactions of a 263-bp region of the *CoxI* gene. These oligonucleotides were based on alignments of mitochondrial DNA sequences from three species of pulmonates with sequence data published in GenBank: *Albinaria coerulea*, *Cepaea nemoralis*, and *Euhadra herklotsi* (accession numbers X83390, U23045, and Z71693, respectively). The complete mitochondrial DNA sequences for *C. nemoralis* and *A. coerulea* were determined previously (Terrett et al. 1996 and Hatzoglou et al. 1995, repectively), while much sequence information exists for *E. herklotsi* (Yamazaki et al. 1997). Primers for the *CoxI* gene were 5′-CTTRTTTTGATTTTTTGGTC-3′ and 5′-GYCAMCTAAAIACTTTAATMCC-3′. PCR amplification was conducted in 50-μL reactions containing 1× PCR buffer (BRL), 2 mM $MgCl_2$, 200 μM dNTPs, 0.4 pmol/μL primers, 1 U Taq DNA polymerase, and 25 ng template DNA. Thermal cycling was performed in an MJ research Mini-cycler using 35 cycles of the following steps: 94°C for 30 s for denaturation, 55°C for 30 s for annealing primers, and 72°C for 30 s for fragment extension. A ~410-bp region of the *Cytb* gene was amplified and sequenced using the universal molluscan primers (U*Cytb*151F and U*Cytb*270R) designed by Merritt et al. (1998). Amplification of this gene was accomplished as above, except that only 0.1 pmol/μL of each primer was used in each reaction and the $MgCl_2$ concentration was increased to 2.5 mM. Thermal cycling was performed using an initial incubation at 94°C for 2 min followed by 40 cycles consisting of: 10 s at 94°C for denaturation, 10 s at 45°C for annealing primers, and 10 s at 72°C for product extension. All PCR products were purified using Qiagen PCR purification spin-columns following the procedure specified by the manufacturer. Sequencing took place using an Applied Biosystems, Inc. Model 377 automated fluorescent sequencer. Reactions were conducted for both strands of the PCR amplicon, using BigDye di-deoxyribonucleotide terminators (Applied Biosystems, Inc.) and following the manufacturer's protocols, except that the annealing temperatures of the sequencing reactions of the *CoxI* and *Cytb* fragments were changed to 55 and 45°C, respectively.

Data analyses

AFLP data

A graphical representation of within- and between-population relationships of individuals was obtained using UPGMA (unweighted pair-group method with arithmetic averaging) cluster analysis, which was performed using NTSYS-pc (Rohlf 1993). This procedure was conducted on the half-matrix of all pairwise genetic similarities of individuals in the data set quantified using the Dice coefficient (Dice 1945).

Additional information about genetic diversity and population structuring of *Oxyloma* in the southwestern U.S.A. was obtained using the program Tools for Population Genetic Analyses (TFPGA) (Miller 1997). Many analyses relied on the use of an allele-frequency estimator described by Lynch and Milligan (1994), which was calculated by TFPGA. Use of this estimator required the assumption that each population was in Hardy–Weinberg equilibrium and that each genetic marker was from a Mendelian-segregating locus. Many cases of land-snail populations displaying Hardy–Weinberg genotypic proportions exist in the literature (for example, McCracken and Brussard 1980; Schilthuizen and Lombaerts 1994), however, other studies have reported cases of extreme heterozygote deficiencies (Boato 1988; Lazaridou-Dimitriadou et al. 1994). For this reason, the assumption of Hardy–Weinberg equilibrium may be unrealistic in this system. All pulmonate snails are hermaphrodites (Tompa 1984), and selfing has been documented in multiple succineid taxa (Patterson 1970; Bayne 1974) including *Oxyloma retusa* and *Oxyloma salleana* (Patterson 1970). The extent that selfing could reduce observed heterozygosity in natural populations of *O. haydeni* is unknown. Because the use of dominant AFLP markers did not permit explicit testing for Hardy–Weinberg genotypic proportions, all allele frequency based analyses were also performed under the assumption that selfing is obligatory in the populations studied, resulting in the presence of individuals that are completely homo-

Table 1. Genetic diversity within the four populations of *Oxyloma haydeni* studied.

Population	H_{HW}	H_{Hom}
Three Lakes (3L)	0.2338*ac*	0.1749*ab*
Indian Gardens (IG)	0.2111*ab*	0.2052*a*
Vaseys Paradise (VP)	0.1648*bc*	0.1184*b*
–9 Mile Spring (–9M)	0.0858	0.0608

Note: H_{HW} refers to the average expected heterozygosity for each population when allele frequencies were estimated assuming Hardy–Weinberg genotypic proportions. H_{Hom} refers to the average expected heterozygosity for each population when allele frequencies were estimated assuming each individual was homozygous at each AFLP locus. Values of H_{HW} or H_{Hom} followed by common letters are not significantly different at the $\alpha = 0.0125$ level.

zygous at all loci examined. This approach provided estimates of within- and among-population genetic variances under two extreme cases (Hardy–Weinberg equilibrium and complete homozygosity), while realizing that the true values are likely somewhere in between those actually obtained from the analyses.

Allele-frequency estimates were used to characterize levels of within-population genetic diversity as calculated by Nei's (1978) unbiased expected heterozygosity (H). Estimates of H were calculated from allele-frequency estimates obtained from the Lynch and Milligan (1994) estimator (H_{HW}) and the complete homozygosity model (H_{Hom}) described above. Tests for differences in H_{HW} or H_{Hom} were performed for each pair of populations using a one-sample t test conducted on the difference in H ($\triangle H$) observed at each locus. These analyses, which were evaluated at the $\alpha = 0.0125$ level to correct the Type I error rate, tested the null hypothesis $\triangle H = 0$.

The distance measure of Nei (1978), appropriate for use in situations where both drift and mutation are in effect (Weir 1996), was applied to each pairwise combination of populations. These distances were also represented graphically using the UPGMA procedure of TFPGA. Additionally, the relative robustness of each node in this four-population dendrogram was evaluated using a bootstrapping procedure over loci (1000 replicates) that recorded the proportion of times that patterns observed in the original UPGMA dendrogram appeared in bootstrap data sets. This analysis was performed under both of the genotype frequency assumption sets described above.

Two statistical methods were used to estimate the proportion of among-population genetic variation. First, Weir and Cockerham's (1984) theta (θ), an unbiased estimator of Wright's (1978) F_{ST}, was calculated by TFPGA and used to test for differences in allele frequencies among the four populations studied. Estimates of θ were obtained for both of the genotype frequency assumption sets described above, resulting in the calculation of θ_{HW} and θ_{Hom}. Ninety-five percent confidence limits of θ estimates were obtained from a bootstrap procedure in which 10 000 new data sets were constructed by resampling with replacement over loci. Confidence limits for θ that did not overlap with 0 (no evidence for differentiation of populations) were interpreted as evidence for significant differences of allele frequencies among populations. Second, an analysis of molecular variance (AMOVA) was conducted using WINAMOVA (Excoffier et al. 1992) to test for the presence of population structure. The distance measure used for this analysis was calculated as distance = 1 – similarity, where the similarity measure was the Dice coefficient referred to above. Variation in marker-phenotype distances was used to calculate Φst, another F_{ST} analog. Departure of the estimate of among-population variation (Φst) from random expectations was evaluated using a permutational test consisting of 1000 replicates. In addition, a Markov chain Monte Carlo variation of Fisher's exact test based on the Metropolis algorithm (Metropolis et al. 1953) was used to test for locus-specific differences in marker-phenotype frequencies (rather than allele frequencies) of the four populations studied. This procedure, described by Raymond and Rousset (1995) and performed by TFPGA, obtained an unbiased estimate of the exact p value. Data for each locus were analyzed as 4 × 2 contingency tables (4 populations and 2 marker states per locus) to test for overall differences in marker-phenotype frequencies at each locus among populations. Additionally, all pairwise combinations of populations were compared at each locus (all possible 2 × 2 tables). Fisher's combined probability test (Fisher 1954; Sokal and Rohlf 1995) was used in both cases to generate global estimates of the significance over loci. P-value estimates for each contingency table were obtained using 25 batches of 2000 permutations per batch (50 000 total permutations) and an initial 1000-step dememorization procedure. The use of batching permitted calculation of standard errors of p-value estimates.

Sequence data

After gels were run, sequences were aligned and edited manually using Sequence Navigator (Applied Biosystems, Inc.). Because of the highly degenerate nature of the *Cytb* primers used, data from 5′ ends of the sequence data were frequently difficult to interpret unambiguously. As a result, only 356 bp of internal sequence information was used for analyses. Aligned and edited sequence data were entered into MegAlign (Lasergene), which was used to create input files for PAUP 3.0 (Swofford 1991). In PAUP, sequence data were analyzed via maximum parsimony using exhaustive searches. Additional support for tree topologies was obtained though the use of a bootstrap procedure consisting of 100 bootstrap replicates.

Results

AFLP patterns

Overall, the AFLP procedure detected 110 scoreable polymorphic markers using five primer combinations. The number of variable fragments ranged from 16 to 28 per primer combination. In all cases, the number of monomorphic DNA fragments from each gel (range 4–10) was less than the number of variable markers.

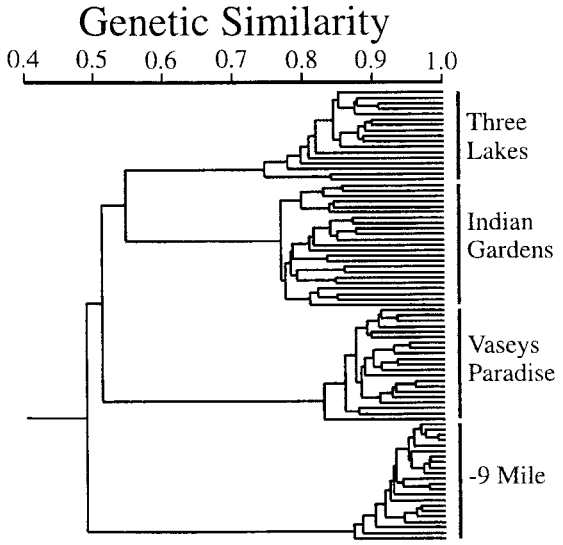
Genetic diversity

Diversity estimates varied for each of the populations studied (Table 1). Based on the analyses, the 3L and IG populations appeared to contain the most genetic variation (3L: $H_{HW} = 0.2338$, $H_{Hom} = 0.1749$; IG: $H_{HW} = 0.2111$, $H_{Hom} = 0.2052$). Diversity estimates did not differ significantly between these two populations. VP contained intermediate levels of diversity ($H_{HW} = 0.1648$, $H_{Hom} = 0.1184$). H_{HW} for this population did not differ from comparable estimates obtained for the 3L and IG populations, however, H_{Hom} was significantly lower in the VP population than in the IG population ($P = 0.0014$). Diversity estimates were lowest at –9M ($H_{HW} = 0.0858$, $H_{Hom} = 0.0608$); both H_{HW} and H_{Hom} were significantly lower for this population than for all the other populations examined.

Population structure

All analyses conducted suggest high levels of differentiation of the four populations studied. The UPGMA dendrogram obtained from the analysis of all individuals in the data set showed that individuals from each population are much more similar to other individuals from that population than

Fig. 2. UPGMA dendrogram of Dice similarity coefficients calculated for the 110 AFLP markers generated from the 83 snails studied. Note that the Three Lakes (3L) and Vaseys Paradise (VP) populations are currently identified as *Oxyloma haydeni kanabensis*, while the Indian Gardens (IG) and –9 Mile Spring (–9M) populations are identified as *O. h. haydeni*.

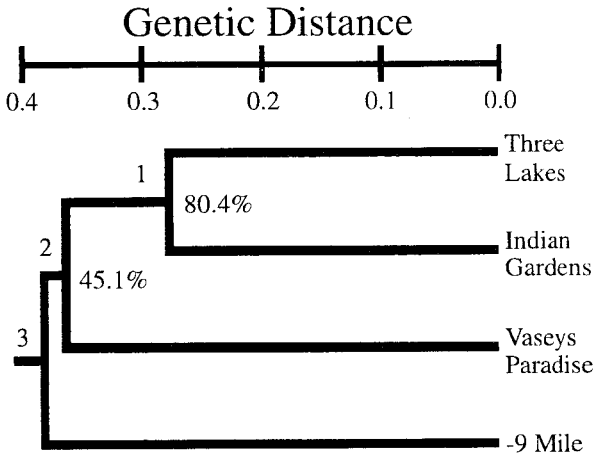


they are to individuals from other populations (Fig. 2). Four main clusters were produced, and while each individual had its own unique AFLP marker profile, snails from the same population invariably clustered together. Additionally, the tree produced by the cluster analysis of distances of each population (Fig. 3) yielded a similar topology. Bootstrap values obtained from this analysis show that the 3L and IG populations clustered together >80% of the time, an unexpected result, considering that these two populations are currently considered to be different subspecies. Genetic distances calculated for each pair of populations ranged from ~0.27 to 0.38 when Lynch and Milligan's (1994) allele-frequency estimator was used. Distances were greater, ranging from 0.36 to 0.48, when individuals were assumed to be homozygous.

Results from *F* statistics and AMOVA analyses provided quantitative evidence to support the UPGMA dendrograms. The overall θ estimates were high and confidence intervals (CIs) did not overlap with 0 (θ_{HW} = 0.59, upper CI = 0.64, lower CI = 0.54; θ_{Hom} = 0.68, upper CI = 0.72, lower CI = 0.64). The estimate of Φst was 0.69 ($p < 0.001$). All the approaches, despite having different assumption sets, indicated that most of the total genetic variation could be accounted for by the partitioning of individuals into populations.

Contingency-table analyses yielded similar results. Analyses of each 4 × 2 table from each locus showed that only 1 of the 110 markers examined did not significantly differ among populations at the α = 0.05 level. Use of Fisher's combined probability test over loci showed highly significant overall differentiation (χ^2 = 2189, df = 220, $p < 0.0001$), a result consistent with the high values observed from *F* statistics and AMOVA analyses. Additionally, results from all pairwise comparisons of populations showed that each population significantly differed in marker frequencies from all other populations (Table 2). Between 55 and 67 markers were significantly different at the α = 0.05 level in comparisons (listed here only as an indicator of differentiation), and overall *p* values were all <0.0001 (χ^2 values ranged from 1076 to 1216, df = 220). In all contingency table analyses, standard errors of *p*-value estimates were <0.005.

Fig. 3. UPGMA dendrogram of Nei's (1978) genetic distances calculated for each pairwise combination of populations in this study. Allele-frequency estimates used in genetic-distance calculations were obtained from the estimator of Lynch and Milligan (1994), which assumed Hardy–Weinberg equilibrium. A comparable analysis performed assuming that individuals were completely homozygous (see text for details) gave the same topology but had greater clustering distances (node 1, 0.3605; node 2, 0.4453; node 3, 0.4581). Note that the Three Lakes and Vaseys Paradise populations contain *Oxyloma haydeni kanabensis* while the Indian Gardens and –9 Mile Spring populations contain *O. h. haydeni*. Percentages associated with nodes refer to the proportion of 1000 bootstrap replicates over loci that resulted in the formation of a cluster similar to ones shown above.



Mitochondrial sequence data

Analysis of the *CoxI* and *Cytb* genes provided 263 and 356 bp of data, respectively. No variation between the two individuals sequenced from each population was detected for the *CoxI* gene, however, considerable variation existed among populations. In total, 14 of the 263 *CoxI* positions (5.3%) were polymorphic (Table 3); all were synonymous differences. GenBank accession numbers for the *CoxI* sequences of the 3L, IG, –9M, and VP samples are AF129471, AF129472, AF129473, and AF129474, respectively. Data on polymorphic nucleotides from the *Cytb* gene are listed in Table 4. The –9M, IG, and VP populations did not vary between the two individuals sequenced from each population, however, a single transition mutation was found between the two individuals sampled from 3L. In total, 23 additional *Cytb* positions (6.5%) varied among populations. Of these polymorphisms, four resulted in changes in polypeptide sequences. In addition, the single nucleotide difference between the two individual samples from 3L also resulted in an amino acid change. GenBank accession numbers for the *Cytb* sequences of the two 3L haplotypes are AF129466 and AF129467 and of the IG, –9M, and VP samples are AF129468, AF129469, and AF129470, respectively.

Table 2. Results from all pairwise analyses of populations using Fisher's exact test.

	Three Lakes				Vaseys Paradise				Indian Gardens				–9 Mile Spring		
	χ^2	df	p	No. of markers	χ^2	df	p	No. of markers	χ^2	df	p	No. of markers	χ^2	df	p
Three Lakes	—	—	—												
Vaseys Paradise	1216	220	<0.0001	60	—	—	—								
Indian Gardens	1076	220	<0.0001	59	1214	220	<0.0001	67	—	—	—				
–9 Mile Spring	1167	220	<0.0001	58	1148	220	<0.0001	55	1204	220	<0.0001	56	—	—	—

Note: P values below the diagonal are the overall results from the use of Fisher's combined probability test over loci. No. of markers are the number of markers that significantly differed in frequency between the two populations at the $\alpha = 0.05$ level (values presented only to serve as an indicator of differentiation).

Table 3. Identities of the 14 polymorphic *CoxI* nucleotides observed out of the 263 sequenced.

	Nucleotide position													
Population	021	057	087	090	114	141	147	159	162	174	180	201	222	240
Three Lakes	C	G	G	A	A	G	A	T	G	A	G	T	T	T
–9 Mile Spring	T	A	–	G	–	–	–	–	–	–	–	–	A	C
Indian Gardens	–	A	A	–	–	–	–	–	–	–	–	–	–	–
Vaseys Paradise	–	A	–	G	G	A	G	A	A	G	A	C	–	C

Note: Dashes indicate the presence of nucleotides identical to those in the Three Lakes individuals.

The single most parsimonious phylogenies for the populations, based on each of the two gene sequences, are shown in Fig. 4. Using the *CoxI* data produced a tree consisting of 14 steps with a consistency index of 1. *Cytb* data generated a phylogeny consisting of 27 steps; the consistency index for this tree was 0.963. The relationships of the populations obtained from the sequence information is in general agreement with the pattern of population similarities elucidated by the UPGMA clustering of the AFLP data. More specifically, both suggest the relatively close relationship of the 3L and IG populations.

Discussion

Within-population genetic variation

The AFLP data suggest the presence of considerable differences in within-population genetic variation among the four populations surveyed (Table 1). One KAS population (3L) and one NAS population (IG) contained the highest levels of diversity, while the NAS population at –9M had significantly less variation than the other three populations examined. Intermediate-diversity estimates were found for the VP population; H_{HW} for this population was not significantly lower than values observed for the populations at 3L and IG, however a significant reduction of H_{Hom} was observed relative to IG.

Multiple explanations can be invoked to account for the patterns of within-population diversity observed in the analyses. Perhaps one main mechanism could involve the actual locations of the populations in question. For example, the lowest levels of diversity were observed at –9M (Table 1), a site immediately adjacent to the Colorado River (Fig. 1) and only a few feet above normal-flow water level. This population is located downslope from the level of the historical mean annual peak flow of 2550 m^3/s that occurred in the Colorado River prior to the construction of the Glen Canyon Dam in 1963 (Sorensen and Kubly 1997). Before dam construction, this location was likely exposed to frequent flooding events that would have completely covered the entire population. These snails generally drown in <8 h in warm water under laboratory conditions and can survive no more than 2–3 days when submerged in cold well-oxygenated water (L.E. Stevens, personal communication). If few –9M individuals survived each flood, then it is plausible that overall genetic diversity was reduced, owing to severe bottlenecks as a result of naturally occurring disturbances. Like –9M, VP is also situated along the Colorado River. Most of the snail habitat at this site, however, is vegetation growing on the vertical surface of the canyon wall well above the river. A 1275 m^3/flood in 1996 destroyed ~16% of the primary ambersnail habitat at VP (Stevens et al. 1997*b*)[3], and while the peak historical flood (8500 m^3/s in 1884; O'Connor et al. 1994) undoubtedly removed much of the snail's habitat, it would not have reached the upper-elevation habitat there. Therefore, bottleneck events as a result of flooding may be responsible for the intermediate levels of genetic diversity seen at Vaseys Paradise, and may play an important role in ambersnail genetic diversity along other streams.

In contrast with those at –9M and VP, the populations at 3L and IG appear to contain high levels of genetic diversity. These are both spring-fed sites that are not subject to large seasonal runoff. Suitable *Oxyloma* habitat at 3L has extended over an area 1.3 km long and up to 90 m wide; population-size estimates have exceeded 100 000 individuals (U.S. Fish

[3] L.E. Stevens, V.J. Meretsky, D.M. Kubly, J.C. Nagy, C. Nelson, J.R. Petterson, F.R. Protiva, and J.A. Sorensen, 1997*b*. The impacts of an experimental flood from Glen Canyon Dam on the endangered Kanab ambersnail at Vaseys Paradise, Grand Canyon, Arizona. Final Report, Grand Canyon Monitoring and Research, Flagstaff, Ariz.

and Wildlife Service 1992*a*). IG contains <150 m^2 of primary habitat and supports considerably fewer individuals (L.E. Stevens, personal communication). The fact that high levels of nuclear genetic variation exist within these two populations suggests that perhaps they have been undisturbed during their relatively recent evolutionary history.

Many studies of terrestrial molluscs have cited facultative or obligate selfing of individuals as a mechanism for reducing genetic diversity in populations (Selander and Hudson 1976; McCracken and Selander 1980; Jarne 1995). Such trends are also well-documented in plants (Schoen and Brown 1991). In statistical analyses, the dominant AFLP markers were interpreted using allele frequencies estimated from models that assumed Hardy–Weinberg equilibrium of populations or the complete homozygosity of individuals. This approach let us obtain ranges of potential values that could have been found in the organisms studied. Genetic diversity is rapidly lost in selfing species (Jarne 1995), and effects can be especially pronounced when relatively few individuals colonize a new population (Selander and Kaufman 1973). In cases where high selfing rates appear to be common, the presence of distinct genetic "strains" of organisms have generally been detected within populations. In this study, however, no two individuals yielded identical AFLP marker profiles (Fig. 2), providing strong evidence that obligate selfing does not occur in the four populations examined. As a result, estimates of within-population genetic variation are likely greater than the estimates of H_{Hom} listed in Table 1. In fact, diversity estimates observed from most populations in this study were generally more comparable with those observed in 23 outcrossing species summarized by Jarne (1995) (outcrossers: average expected gene diversity = 0.130; mixed maters: average expected gene diversity = 0.130; selfers: average expected gene diversity = 0.04). The population at –9M, however, had diversity estimates closer to typical selfing species (H_{HW} = 0.0858, H_{Hom} = 0.0608). At this point, the possibility that differences in reproductive strategy exist among populations cannot be ignored; the –9M population perhaps contains a high proportion of facultative selfers. Future genetic analyses relying on the use of codominant markers would likely assist in making such inferences, as would experiments designed to assess the potential for selfing in these natural populations.

It should be noted that the studies included in Jarne's (1995) review were based on isozyme techniques. Currently there are insufficient data to compare the results of protein-based genetic markers with the AFLP markers employed in this study. The range of diversity values possible for a locus, however, is a function of the number of alleles present at that locus. In Jarne's (1995) review, outcrossers averaged 1.6 alleles per locus per population; this is in accordance with the conventional treatment of dominant markers as simple two-allele systems (Lynch and Milligan 1994; Weir 1996).

Genetic differentiation of populations

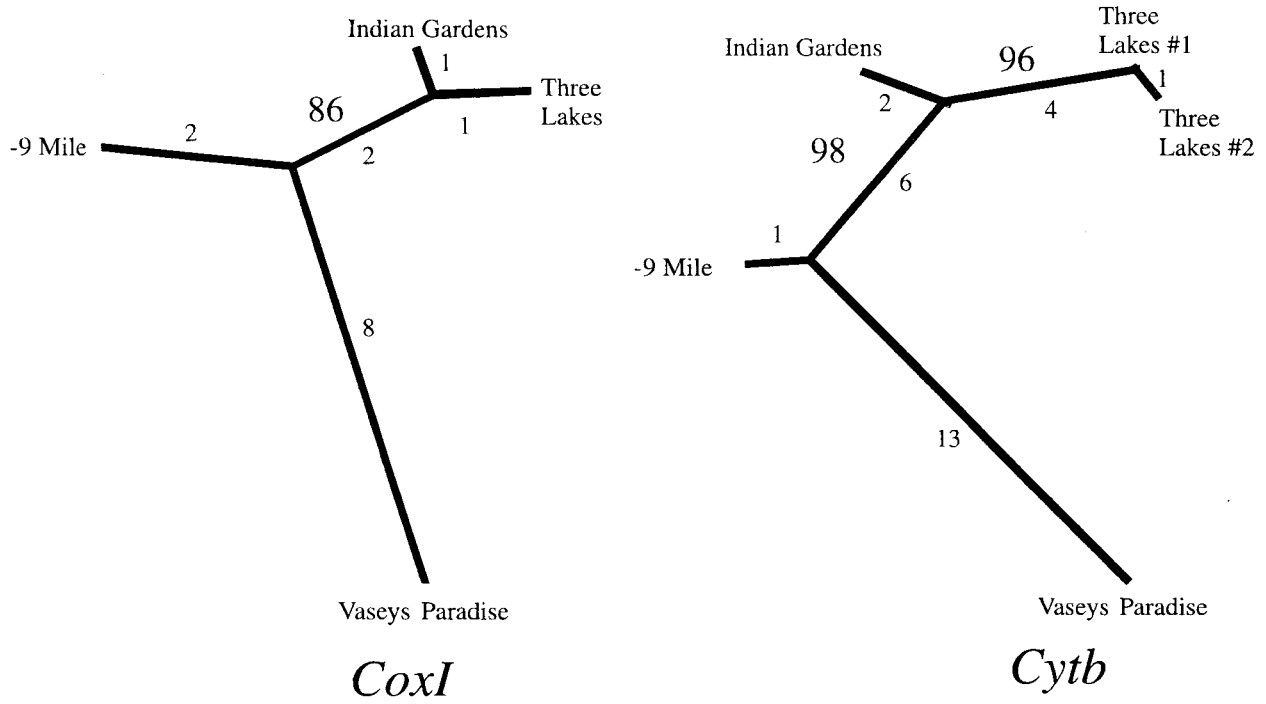
Estimates of genetic structure, as quantified through the use of *F* statistics, AMOVA, and exact tests, suggest that population differentiation is extensive. The estimates of among-population variation (θ_{HW} = 0.59, θ_{Hom} = 0.68, Φst = 0.69) are all in excess of five of the six terrestrial slug species studied by Daniell (1994). F_{ST} estimates from this report

Table 4. Identities of the 24 polymorphic *Cytb* nucleotides observed out of the 356 sequenced.

	Nucleotide position																							
Population	006	023	048	054	075	093	**105**	**136**	**170**	171	**191**	192	216	246	264	285	**291**	309	315	339	342	345	351	354
Three Lakes	C	G	A	A	T	C	A	T	S	C	G	C	T	A	T	T	A	T	G	G	C	T	C	C
–9 Mile Spring	–	–	–	–	C	T	–	G	C	T	A	T	–	–	–	–	G	C	–	–	T	A	–	T
Indian Gardens	–	–	–	–	–	–	–	G	C	–	–	T	–	–	–	–	–	C	–	A	T	–	–	–
Vaseys Paradise	T	C	G	T	C	T	T	G	C	T	A	T	C	T	C	C	–	–	A	–	T	C	T	T

Note: Dashes indicate the presence of nucleotides identical to those seen in the Three Lakes individuals. The S at position 170 indicates the presence of a guanine or cytosine residue. The numbered positions in boldface type code for amino acid changes: position 105, A–T mutation changes threonine to leucine; position 136, T–G mutation changes leucine to valine; position 170, G–C mutation changes threonine to serine; position 191, G–A mutation changes serine to asparagine; position 291, A–G mutation changes threonine to leucine.

Fig. 4. Unrooted phylogenetic trees generated from the parsimony analysis of two mitochondrial genes. The 263 bp of sequence from the *CoxI* gene produces a 14-step tree with a consistency index of 1.0. The 362 bp of sequence from the *Cytb* gene produces a 27-step tree with a consistency index of 0.963. Branch lengths from each tree are given. Numbers in large type are bootstrap values obtained from 100 bootstrap replicates.



ranged from 0.19 to 0.66. Other studies of land snails have reported F_{ST} values of 0.065 and 0.116 for two *Mesomphix* species (Stiven 1989), 0.301 for *Theba pisana* (Johnson 1988), 0.535 for distant populations of *Albinaria corrugata* (Schilthuizen and Lombaerts 1994), 0.128 for *Helix aspersa* (Lazaridou-Dimitriadou et al. 1994), and a range of 0.0767–0.5675 in six species of *Chondrinidae* (Boato 1988). Overall, it is apparent that terrestrial molluscs display a wide variety of genetic-differentiation patterns.

Jarne (1995) summarized patterns of among-population genetic heterogeneity from 31 studies of land snails using Nei's (1987) G_{ST}, another F_{ST} analog. Here, selfers had average G_{ST} values of 0.775 while outcrossing species had average G_{ST} values of 0.231. Species classified as mixed maters had average among-population diversity estimates of 0.447. While estimates of among-population variation in southwestern ambersnails appear to be higher than those seen in other outcrossing terrestrial molluscs, it is important to consider that F_{ST}, θ, and Φst values may not be comparable among studies, simply because computational methods (see for example Holsinger 1999) and genetic markers can differ. Additionally, population distances and locations vary among studies. However, if taken at face value, the estimates of among-population genetic variation in southwestern *Oxyloma* appear to exceed typical values observed in outcrossing land snail populations.

Illustrative of the high levels of differentiation are the results of per-locus Fisher's exact tests. In these analyses, all but one of the 110 markers surveyed significantly differed in frequency among populations. In the analysis of all pairwise combinations of populations, between 55 and 67 markers (50–61%) differed in frequency in pairs of contrasts (Table 2). Overall, these findings suggest long-term separation of the four populations, perhaps since the Pleistocene Epoch. If so, these populations are relics of what might have been a greatly expanded range in more mesic times. Alternately, this pattern could also have been produced by a scenario in which relatively few individuals initially colonized some or all of the sites in more recent times. The Grand Canyon region was extensively surveyed for *Oxyloma* populations between 1991 and 1996 (Sorensen and Kubly 1997, 1998; Stevens et al. 1997*a*, see footnote 2), and no additional populations were discovered that could have acted as sources. However, the formation of Lake Powell upriver from Glen Canyon Dam destroyed vast quantities of riparian habitat on the Colorado River upstream of –9M and VP. Ambersnail populations may have existed previously in these upstream reaches and provided colonists for downstream sites.

Relationships of populations

The analyses of both nuclear AFLP and mitochondrial sequence data yielded the unexpected finding that the taxonomy of southwestern *Oxyloma* may be incorrect. Specifically, the KAS-3L population and the NAS-IG population appear to be more similar than the KAS-3L and KAS-VP populations (Figs. 2–4). While a formal investigation has not yet been undertaken, morphological examinations of KAS have suggested that this subspecies is distinct from *O. h. haydeni* and that KAS deserves specific rank status (Pilsbry 1948; Harris and Hubricht 1982; Spamer and Bogan 1993). The

genetic data, however, strongly suggest that taxonomic criteria distinguishing KAS from NAS require re-evaluation. Currently, nomenclature is based mainly on differences in the penial complex of these organisms. Both the nuclear AFLP markers and mitochondrial sequence data, however, indicate that the KAS-3L and NAS-IG populations are more closely related than the two protected KAS populations currently designated as endangered (3L and VP), suggesting that this anatomical character is phylogenetically misleading. Morphology has long been a source of confusion in molluscan systematics (see for example Williams and Mulvey 1997). In fact, genetic data have provided strong evidence in at least one other case that a reliance on reproductive anatomy (specifically phallus shape) has led to a species of land snail being placed in the wrong genus (Johnson et al. 1986).

Application of AFLP and sequence data for mollusc conservation and management

This study relied on the use of both highly variable AFLP markers and conserved mitochondrial gene sequences. The former provided estimates of within-population genetic diversity and estimates of population structure, and posed possible relationships for the four populations investigated. The mitochondrial sequence data provided an alternate approach for characterizing population-level evolutionary relationships.

Extensive debate has taken place concerning the definition of units for conservation efforts (see, for example, Moritz 1994; Waples 1998; Paetkau 1999; Taylor and Dizon 1999). Regardless of definition, this study showed that each population is genetically distinct and that, assuming long-term separation of populations, each may warrant individual protected status. Without question, extensive differences in nuclear-allele frequencies exist among populations, and limited sequencing of the mitochondrial genome also suggested such patterns. Currently, only the KAS populations at VP and 3L are protected as endangered species. The –9M and IG NAS populations are not federally listed, an unfortunate situation considering this taxon's restricted distribution and habitat in the southwestern U.S.A. Furthermore, the habitats of all four populations are, at least in part, at risk from human impacts. The IG population, however, apparently has not suffered from the extensive modification of its habitat as part of a National Park Service campground. Perhaps sustainable *Oxyloma* populations can persist under relatively high levels of anthropogenic disturbance provided that suitable habitat is maintained.

Based on these findings, a genus-wide revision of North American *Oxyloma* based on molecular data may be required to completely address the endangered status of ambersnails in the southwestern U.S.A. In addition, a formal phylogenetic treatment of morphological characters in *Oxyloma* would appear to be in order. At this time, it appears that the Endangered Species Act may, in fact, be arbitrarily applied to 3L and VP snails.

Acknowledgements

Mr. Brandt A. Child kindly permitted collections of Kanab ambersnails for genetic analysis from the Three Lakes, Utah, population. Assistance with field collections was provided by Debra Bills, Alison Bowden, Mike Huckaby, Jerry Landye, MariAnn MacKenzie, Vicky Meretsky, Clay Nelson, Jodi Niccum, Jim Petterson, and Mike Venditto. Russel Vossbrink provided valuable laboratory assistance. Funding for this research was provided by the Arizona Game and Fish Department, with coordination and administrative support by Dennis M. Kubly. Collection permits were authorized by the National Park Service and U.S. Fish and Wildlife Service. Thierry Backeljau provided excellent comments on this work that greatly improved the quality of the manuscript.

References

Bayne, C.J. 1974. Physiology of the pulmonate reproductive tract: location of spermatozoa in isolated, self-fertilizing succinid snails. Veliger, **16**: 169–175.

Boato, A. 1988. Microevolution in *Solatopupa* landsnails (Pulmonata Chondrinidae): genetic diversity and founder effects. Biol. J. Linn. Soc. **34**: 327–348.

Clarke, A.H. 1991. Status survey of selected land and freshwater gastropods in Utah. U.S. Fish Wildl. Serv. Rep.

Daniell, A. 1994. Genetics and terrestrial mollusc conservation. Mem. Queensl. Mus. **36**: 47–53.

Dice, L.R. 1945. Measures of the amount of ecologic association between species. Ecology, **26**: 297–302.

Excoffier, L., Smouse, P.E., and Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics, **131**: 479–491.

Fisher, R.A. 1954. Statistical methods for research workers. 12th ed. Oliver and Boyd, Edinburgh.

Harris, S.A., and Hubricht, L. 1982. Distribution of the species of the genus *Oxyloma* (Mollusca, Succineidae) in southern Canada and the adjacent portions of the United States. Can. J. Zool. **60**: 1607–1611.

Hatzoglou, E., Rodakis, G.C., and Lecanidou, R. 1995. Complete sequence and gene organization of the mitochondrial genome of the land snail *Albinaria coerulea*. Genetics, **140**: 1353–1366.

Holsinger, K.E. 1999. Analysis of genetic diversity in geographically structured populations: a Bayesian perspective. Hereditas, **130**: 245–255.

Jarne, P. 1995. Mating systems, bottlenecks, and genetic polymorphism in hermaphroditic animals. Genet. Res. (Camb.), **65**: 193–207.

Johnson, M.S. 1988. Founder effects and geographic variation in the land snail *Theba pisana*. Heredity, **61**: 133–142.

Johnson, M.S., Murray, J.J., and Clarke, B.C. 1986. High genetic similarities and low heterozygosities in land snails of the genus *Samoana* from the Society Islands. Malacologia, **7**: 97–106.

Lazaridou-Dimitriadou, M., Karakousis, Y., and Staikou, A. 1994. Geographical variation in shell morphology and isoenzymes of *Helix aspersa* Müller, 1774 (Gastropoda, Pulmonata), the edible land snail, from Greece and Cyprus. Heredity, **72**: 23–35.

Lynch, M., and Milligan, B.G. 1994. Analysis of population genetic structure with RAPD markers. Mol. Ecol. **3**: 91–99.

McCracken, G.F., and Brussard, P.F. 1980. The population biology of the white-lipped land snail, *Triodopsis albolabris*: genetic variability. Evolution, **34**: 92–104.

McCracken, G.F., and Selander, R.K. 1980. Self-fertilization and monogenic strains in natural populations of terrestrial slugs. Proc. Natl. Acad. Sci. U.S.A. **77**: 684–688.

Merritt, T.J.S., Shi, L., Chase, M.C., Rex, M.A., Etter, R.J., and Quattro, J.M. 1998. Universal cytochrome *b* primers facilitate intraspecific studies in molluscan taxa. Mol. Mar. Biol. Biotechnol. **7**: 7–11.

Metropolis, N., Rosenbluth, A.W., Rosenbluth, M.N., Teller, A.H., and Teller, E. 1953. Equation of state calculations by fast computing machines. J. Chem. Phys. **21**: 1087–1092.

Miller, M.P. 1997. Tools for population genetic analyses (TFPGA): a Windows program for the analysis of allozyme and molecular population genetic data. [Available from the author: M.P. Miller, Northern Arizona University, Flagstaff.]

Moritz, C. 1994. Defining 'Evolutionary Significant Units' for conservation. Trends Ecol. Evol. **9**: 373–375.

Müllenbach, R., Lagoda, P.J.L., and Welter, C. 1989. An efficient salt–chloroform extraction of DNA from blood and tissues. Trends Genet. **5**: 391.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, **89**: 583–590.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, New York.

O'Connor, J.E., Ely, L.L., Wohl, E.E., Stevens, L.E., Melis, T.S., Kale, V.S., and Baker, V.R. 1994. A 4,500-year record of large floods on the Colorado River in the Grand Canyon, Arizona. J. Geol. **102**: 1–9.

Paetkau, D. 1999. Using genetics to identify intraspecific conservation units: a critique of current methods. Conserv. Biol. **13**: 1507–1509.

Patterson, C.M. 1970. Self-fertilization in the land snail family Succineidae. J. Conchol. **108**: 61–62.

Pilsbry, H.A. 1948. Land Mollusca of North America. Acad. Nat. Sci. Phila. Monogr. No. 2. pp. 521–1113.

Pilsbry, H.A., and Ferriss, J.H. 1911. Mollusca of the southwestern states, V: the Grand Canyon and northern Arizona. Proc. Acad. Nat. Sci. Phila. **63**: 174–199.

Raymond, M.L., and Rousset, F.R. 1995. An exact test for population differentiation. Evolution, **49**: 1280–1283.

Rohlf, F.J. 1993. NTSYS-pc: numerical taxonomy and multivariate analysis system, verson 1.8. Exeter Software, Setauket, N.Y.

Schilthuizen, M., and Lombaerts, M. 1994. Population structure and levels of gene flow in the Mediterranean land snail *Albinaria corrugata* (Pulmonata: Clausiliidae). Evolution, **48**: 577–586.

Schoen, D.J., and Brown, A.H.D. 1991. Intraspecific variation in population gene diversity and effective population size correlates with the mating system in plants. Proc. Natl. Acad. Sci. U.S.A. **88**: 4494–4497.

Selander, R.K., and Hudson, R.O. 1976. Animal population structure under close inbreeding: the land snail *Rumina* in southern France. Am. Nat. **110**: 695–718.

Selander, R.K., and Kaufman, D.W. 1973. Self-fertilization and genetic population structure in a colonizing land snail. Proc. Natl. Acad. Sci. U.S.A. **70**: 1186–1190.

Sokal, R., and Rohlf, F.J. 1995. Biometry. 3rd ed. W.H. Freeman and Co., New York.

Sorensen, J.A., and Kubly, D.M. 1997. Investigations of the endangered Kanab ambersnail: monitoring, genetic studies, and habitat evaluation in Grand Canyon and northern Arizona. Tech. Rep. No. 122 of the Nongame and Endangered Wildlife Program, Arizona Game and Fish Department, Phoenix, Ariz.

Sorensen, J.A., and Kubly, D.M. 1998. Monitoring and habitat surveys of the endangered Kanab ambersnail in Grand Canyon and northern Arizona. Tech. Rep. No. 125 of the Nongame and Endangered Wildlife Program, Arizona Game and Fish Department, Phoenix, Ariz.

Spamer, E.E., and Bogan, A.E. 1993. Mollusca of the Grand Canyon and vicinity, Arizona: new and revised data on diversity and distributions, with notes on Pleistocene–Holocene mollusks of the Grand Canyon. Proc. Acad. Nat. Sci. Phila. **144**: 21–68.

Stiven, A.E. 1989. Population biology of two land snails (*Mesomphix* spp.): variation among six southern Appalachian sites with differing disturbance histories. Oecologia, **79**: 372–382.

Swofford, D.L. 1991. PAUP: phylogenetic analysis using parsimony, version 3.0. Illinois Natural History Survey, Champaign.

Taylor, B.L., and Dizon, A.E. 1999. First policy then science: why a management unit based soley on genetic criteria cannot work. Mol. Ecol. **8**: S11-S16.

Terrett, J.A., Miles, S., Thomas, R.H. 1996. Complete DNA sequence of the mitochondrial genome of *Cepaea nemoralis* (Gastropoda: Pulmonata). J. Mol. Evol. **42**: 160–168.

Tompa, A.S. 1984. Land snails (Stylommatophora). *In* The Mollusca. Vol. 7. Reproduction. *Edited by* A.S. Tompa, N.H. Verdonk, and J.A.M. Van Den Biggelaar. Academic Press, Orlando. pp. 47–140.

Travis, S.E., Maschinski, J., and Keim, P. 1996. An analysis of genetic variation in *Astragalus cremnophylax* var. *cremnophylax*, a critically endangered plant, using AFLP markers. Mol. Ecol. **5**: 735–745.

U.S. Fish and Wildlife Service. 1991*a*. Endangered and threatened wildlife and plants; emergency rule to list the Kanab ambersnail as endangered. Fed. Regist. **56**: 37 668 – 37 671.

U.S. Fish and Wildlife Service. 1991*b*. Endangered and threatened wildlife and plants; proposal to list the Kanab ambersnail as endangered and designate critical habitat. Fed. Regist. **56**: 58 020 – 58 026.

U.S. Fish and Wildlife Service. 1992*a*. Endangered and threatened wildlife and plants; final rule to list the Kanab ambersnail as endangered. Fed. Regist. **57**: 13 657 – 13 661.

U.S. Fish and Wildlife Service. 1992*b*. Kanab ambersnail (*Oxyloma haydeni kanabensis*). Endangered Species Technical Bulletin No. 17. U.S. Fish and Wildlife Servie, Endogenered Species Program, Washington, D.C. p. 6.

U.S. Fish and Wildlife Service. 1995. Kanab ambersnail (*Oxyloma haydeni kanabensis*) Recovery Plan. [Available from U.S. Fish and Wildlife Service Region 6 / Region 2, Salt Lake City, Utah.]

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. **23**: 4407–4414.

Waples, R.S. 1998. Evolutionary significant units, distinct population segments, and the Endangered Species Act: reply to Pennok and Dimmik. Conserv. Biol. **12**: 718–721.

Weir, B.S. 1996. Genetic data analysis II: methods for discrete population genetic data. Sinauer Associates, Inc., Sunderland, Mass.

Weir, B.S., and Cockerham, C.C. 1984. Estimating *F*-statistics for the analysis of population structure. Evolution, **38**: 1358–1370.

Williams, J.D., and Mulvey, M. 1997. Recognition of freshwater mussel taxa: a conservation challenge. *In* Principles of conservation biology. 2nd ed. *Edited by* G.K. Meffe and C.R. Carrol. Sinauer Associates, Inc., Sunderland, Mass. pp. 64–65.

Wright, S. 1978. Evolution and the genetics of populations. Vol. 4. Variability within and among natural populations. University of Chicago Press, Chicago.

Yamazaki, N., Ueshima, R., Terrett, J.A., Yokobori, S., Kaifu, M., Segawa, R., Kobayashi, T., Numachi, K., Ueda, T., Nishikawa, K., Watanabe, K., and Thomas, R.H. 1997. Evolution of pulmonate gastropod mitochondrial genomes: comparisons of gene organizations of *Euhadra*, *Cepaea*, and *Albinaria* and implications of unusual tRNA secondary structures. Genetics, **145**: 749–758.